Fluorimetry Assay Optimization Suggestions

Microplates

If using 96-well plates, use '½-area' plates. Wells are half the diameter, which increases the depth of the reaction mixture and improves signal consistency.

White vs. black

White and black plates differ in their reflective properties. Black plates absorb light, reducing background and cross-talk; white plates reflect light, maximizing light output signal. Fluorescent assays generally employ black plates. However, as the number of wells-per-plate (plate density) increases, the assay volume decreases, which means less fluorophore per well. Thus, for 384-well and up, it may be advantageous to use white plates to maximize signal. This must be determined empirically.

Fluorimetry with cells

Typically, clear bottom plates are used for fluorescent microscopy, but if performing fluorimetry (measuring overall fluorescence in well from above) with cells, we suggest the use of all-black plates, unless you have equipment/ software (e.g., BioTek® Cytation™ 5 Cell Imaging Multi-Mode Reader and Gen5™ Data Analysis Software) that can subtract out plate background. Clear bottom plates can be temporarily converted to opaque plates by application of a sheet to the bottom of the plate (e.g., Perkin Elmer's BackSeal™ Adhesive Bottom Seal). The color of the bottom sheet should match the color of the sides of the plate wells (black or white). *Contact us at info@enziumlabs.com for more tips on fluorimetry with cells (or conditioned cell media).*

Setting up the assay

Minimize exposure of EnSens® Far Red Fluorogen to light.

Proteases tend to be autolytic (self-degrading). Always aliquot samples containing protease, minimize time thawed, and store on ice while thawed.

Excitation and emission filters or wavelengths

Maximal EnSens® Far Red Fluorogen excitation/emission wavelengths are Ex 635/ Em 656 nm, but Ex 610-649 / Em 655-690 nm can be used. Use wavelengths that are closest to the maximal values and farthest from each other. For example, 649nm excitation and 655nm emission (with 5nm bandwidth) will give poor results compared to 635nm excitation and 665nm emission (larger difference in excitation and emission wavelengths). If you do use wavelengths that

are farther apart, experiment with wider bandwidth (see below). Note: There is an alternate excitation peak at 470nm which might be used, although it is much weaker than the excitation peak at 635nm.

Bandwidth/Bandpass/Slit Width

EnSens® excitation and emission wavelengths are relatively close, so recommended bandwidth is 5-10nm (2.5-5nm to either side of the excitation/emission wavelengths). If you are using wavelengths that are farther apart, experiment with wider bandwidths (20-40nm), as long as the excitation/emission wavelengths, including bandwidths, do not overlap.

Gain/sensitivity and Z-position/Z-height settings

If gain/sensitivity is set too low, the 'window' of detection is very small, cramping the assay RFU values together. Depending on your fluorimeter's software, you may be able to choose "scale to high well" or "optimal gain", keeping in mind that it may set the gain too low to compensate for the brightness of the positive control substrate (EnSens®-PA). *Typically, the gain setting for EnSens substrates will be 2-3X higher than for FRET substrates.*

We suggest an initial read with just your positive controls (a well with EnSens®-PA; and a well with purified enzyme), taking reads at different gain settings. If you know your fluorimeter's maximum RFU capacity, find a gain that results in EnSens®-PA RFU that are 50-100% of that maximum. This should provide a good window for detecting protease activity in your samples. For example, if your fluorimeter's maximum RFU is 60,000, choose a gain setting that gives you 30,000 -60,000 RFU for EnSens®-PA (substrate positive control), and 3000-10,000 RFU for EnSens® substrate with purified enzyme (enzyme positive control). If your samples have very low protease activity, you may need to set gain at maximum.

Most fluorimeters also have a way to automatically set the Z-position (the point in the well at which it focuses to take the read) based on its read of a well assigned by you (a well containing EnSens®-PA). This may or may not be necessary, but it does optimize the read.

Reaction mixtures:

Set up all reaction conditions in duplicate or triplicate. Set up unknown samples in several dilutions.

Equilibrate all assay components to assay temperature shortly before combining them to start the assay.

When measuring activity in biological samples, if at all possible include positive control wells with 'known' active protease (purified enzyme). In order for the assay to yield quantitative results, a standard curve of 'known' active protease must be run each time a plate is read. Proteases tend to be autolytic (self-degrading). Always aliquot samples containing protease, minimize time thawed, and store on ice while thawed.

Use at least the minimum reaction mixture volume set by the fluorimeter manufacturer, and maintain uniform reaction volumes—changes in volume will result in signal inconsistency.

Thoroughly mix reactions, but avoid or pop (with needle or pipet tip) bubbles. If using 96-well plates, tap plate to overcome surface tension and ensure that the reaction mixture covers the entire well bottom. Any of these can result in signal inconsistency.

Depending on assay conditions, there may be a lag in activity lasting from 5 to 30 minutes. In order to determine optimal assay period, the initial assay should be kinetic if possible; e.g., reads every minute for two hours.

As suggested, the final concentration of EnSens® substrate in the assay is 200nM. If greater sensitivity is needed, use the substrate at higher concentration (e.g. 500nM); keep in mind this may increase background.